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**Genes: Structure and Regulation:**  
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## Calcium-dependent Activation of Interleukin-21 Gene Expression in T Cells\*

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Interleukin (IL)-21 is a  $\gamma_c$ -dependent cytokine produced by activated T cells with important actions for T, B, and NK cells. The IL-21 gene is adjacent to the IL-2 gene, and like IL-2, IL-21 is strongly induced at the transcriptional level after T cell activation. Interestingly, however, in contrast to the IL-2 gene, a calcium ionophore alone was sufficient to induce IL-21 gene expression in preactivated T cells. Two DNase I hypersensitivity sites were found in the IL-21 gene, corresponding to nucleotide sequences that are conserved in humans and mice. One site is located at the IL-21 promoter region and conferred T cell receptor-mediated IL-21 gene transcription. TCR-induced IL-21 gene expression was inhibited by cyclosporin A and FK506. Correspondingly, the IL-21 5'-regulatory region contains three NFAT binding sites, and induction of IL-21 promoter activity was impaired when these sites were mutated or following treatment with cyclosporin A. Thus, our studies reveal that in contrast to IL-2, a calcium signal alone is sufficient to mediate induction of the IL-21 in preactivated T lymphocytes and that this induction appears to result from specific NFAT binding.

Interleukin (IL)-21 is a type I cytokine whose receptor is most closely related to the IL-2 receptor  $\beta$  chain and is expressed on T, B, and NK cells (1–3). Like IL-2, IL-4, IL-7, IL-9, and IL-15, IL-21 utilizes the common cytokine receptor  $\gamma$  chain ( $\gamma_c$ ) (1, 4, 5). Analogous to other cytokines that utilize  $\gamma_c$ , IL-21 activates Jak1 and Jak3 to initiate signal transduction, but unlike other  $\gamma_c$ -dependent cytokines, which predominantly activate Stat5 proteins (IL-2, IL-7, IL-9, and IL-15) or Stat6 (IL-4), IL-21 preferentially activates Stat1 and Stat3 (2, 4). IL-21 is produced by activated CD4<sup>+</sup> T cells and affects the growth, survival, and functional activation of lymphocytes (3). IL-21 acts *in vitro* as a co-mitogen for anti-CD3-induced thymocyte and peripheral T cell proliferation (3), it augments NK cell expansion and differentiation from human CD34<sup>+</sup> cells when cultured with IL-15 and Flt-3 ligand (3), and it activates NK-cytolytic activity (3, 6).

In addition to its effects on T cells, IL-21 can enhance the

proliferative response of human and murine B cells stimulated *in vitro* with antibodies to CD40, but it inhibits B cell proliferation in response to anti-IgM + IL-4 (3) and can augment B cell death (7, 8). Within the B cell lineage, IL-21 critically regulates immunoglobulin (Ig) production and cooperates with IL-4 for the production of multiple antibody classes *in vivo* (9). IL-21R<sup>−/−</sup> mice have markedly diminished IgG1 but greatly elevated IgE levels in response to antigen (9), and correspondingly, IL-21 can inhibit antigen-induced IgE production (10). Mice lacking expression of both IL-21R and IL-4 exhibit a dysgammaglobulinemia with severely impaired IgG and IgE responses (9). IL-21 differentially influences B cell fate depending on the signaling context. Although IL-21 induces the death of resting B cells (7), it promotes differentiation of B cells activated in a T cell-dependent manner into memory and plasma cells, explaining how IL-21 can be proapoptotic for B cells *in vitro* yet critical for antigen-specific immunoglobulin production *in vivo* (8).

In T cells, IL-21 can cooperate with IL-15 or IL-7 to drive the expansion of T cells, particularly of CD8<sup>+</sup> T cells (11). This effect correlates with the ability of IL-21 to promote cytolytic activity and anti-tumor effects (11). IL-21 has also been reported to act on NK cells. One report indicated that it can increase NK cell maturation, whereas another suggested it can inhibit NK cell expansion (3, 6). To better understand the basis for regulation of this critical cytokine, we now have studied the regulatory elements controlling expression of this gene.

### MATERIALS AND METHODS

**Mice and Cell Culture**—C57BL/6 mice were obtained from the Jackson Laboratory. NFATc2 knock-out mice were a gift from L.H. Glimcher (Harvard School of Public Health, Boston, MA). Mice used in these experiments were 8–10 weeks of age. All experiments were performed under protocols approved by the National Institutes of Health Animal Use and Care Committee and followed the NIH guidelines "Using Animals in Intramural Research." Single-cell suspensions from the spleen were prepared and cultured at  $1 \times 10^6$ /ml in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin G, and 100  $\mu$ g/ml streptomycin. CD4<sup>+</sup> T cells were isolated from the spleens by positive selection with MACS purification (Miltenyi Biotech). These isolated CD4<sup>+</sup> T cells were activated by incubating in the presence of 2  $\mu$ g/ml of anti-CD3 and 2  $\mu$ g/ml of anti-CD28 mAbs (PharMingen) for 2 days and expanded in the presence of 100 units/ml of IL-2 for another day.

**5'-RACE**—5'-RACE was performed using the GeneRacer kit (Invitrogen). Total cellular RNA isolated from activated CD4<sup>+</sup> T cells was dephosphorylated using calf intestinal alkaline phosphatase, and the 5'-"cap" of mRNA was removed using tobacco acid pyrophosphatase. An RNA oligonucleotide was then ligated to the full-length, decapped mRNAs, and reverse transcription was performed using oligo(dT) as a primer. The resultant cDNAs were PCR-amplified using the GeneRacer 5'-primer and a primer specific for the IL-21 gene (5'-TTCAGCTGTCAACAATGTC-3'). The GeneRacer 5'-nested primer and the same IL-21 primer were then used in a second, nested PCR.

**Quantitative Real Time PCR**—CD4<sup>+</sup> splenocytes isolated from C57BL/6 mice were activated with anti-CD3 and anti-CD28 for 2 days,

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<sup>1</sup> The abbreviations used are: IL, interleukin; RACE, rapid amplification of cDNA ends; WT, wild type; EMSA, electrophoretic mobility shift assay; HS, hypersensitivity; 6-FAM, 6-carboxyfluorescein; TAMRA, carboxytetramethylrhodamine; CsA, cyclosporin A; TCR, T cell receptor.

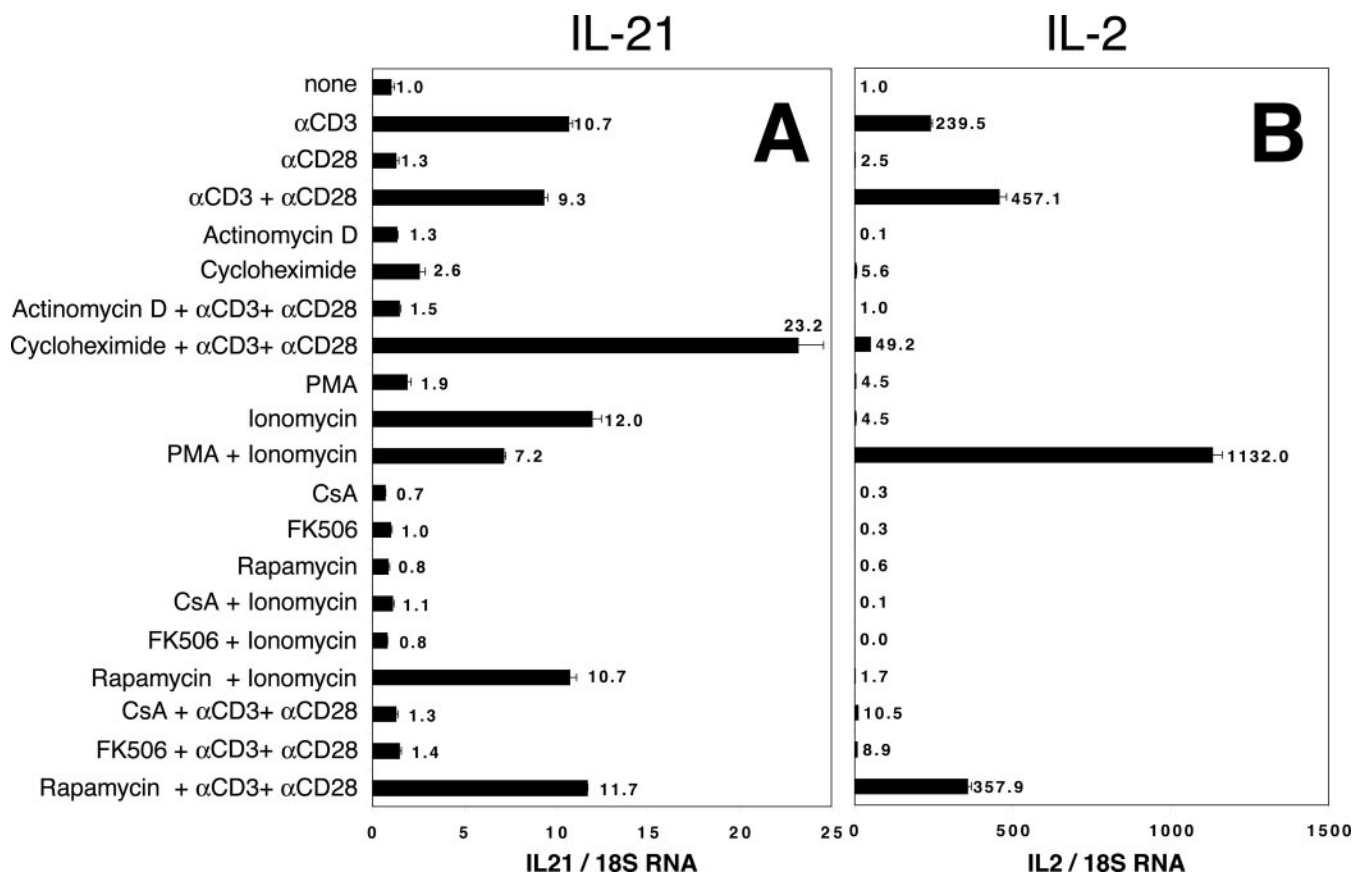


FIG. 1. **Quantitative real time PCR analysis of IL-21 expression in murine T lymphocytes.** Murine T lymphocytes were activated with anti-CD3 + anti-CD28 ( $\alpha$ CD3 +  $\alpha$ CD28) mAbs for 2 days, washed, and then expanded for another day. Cells were then either not stimulated or cultured in the presence of several different stimuli for 4 h, and IL-21 (A) and IL-2 (B) mRNA levels were analyzed by real time PCR. Where indicated, inhibitors (actinomycin D, cycloheximide, CsA, FK506, and rapamycin) were added 30 min before treatment with anti-CD3, anti-CD28, phorbol 12-myristate 13-acetate (PMA), or ionomycin. For each sample, the bar represents the mean ratio  $\pm$  S.E. of IL-21 (A) or IL-2 (B) mRNA to that of 18 S ribosomal mRNA.

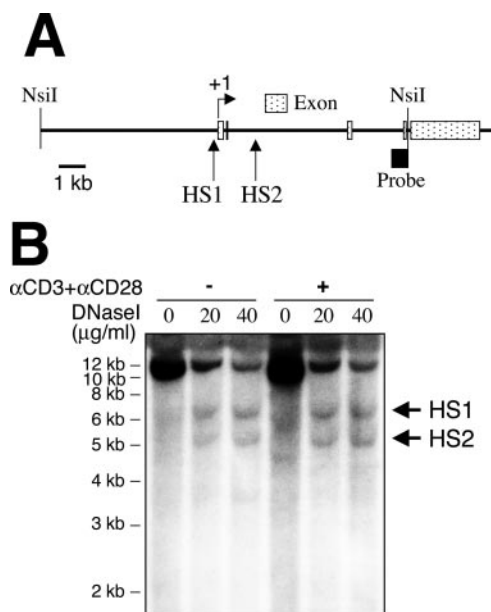


FIG. 2. **Characterization of DNase I hypersensitive sites of the mouse IL-21 gene.** A, schematic showing locations of HS1 and HS2 in the mouse IL-21 gene. The thick bar represents the probe; the five exons of the gene are shown. B, nuclei were isolated from preactivated T lymphocytes cultured for 1 h in the absence or presence of anti-CD3 + anti-CD28 ( $\alpha$ CD3 +  $\alpha$ CD28) and then digested with DNase I as indicated. DNA was extracted, digested with NsiI, and analyzed on 0.6% agarose gels. HSs were mapped by hybridizing NsiI-digested DNA with the probe indicated in A.

and these activated cells were washed and then cultured in the presence of IL-2 for 1 day. Total RNA was isolated from these preactivated CD4 T cells using TRIzol (Invitrogen). First-strand cDNAs were made using the Omniscript RT Kit (Qiagen). Quantitative real time PCR was performed on a 7900H sequence detection system (Applied Biosystems). The sequences of the primers and TaqMan probes were as follows: murine IL-21, 5'-AAGATTCCTGAGGATCCGAGAAG-3', 5'-GCATTCGTGAGCGTCTATAGTGTGTC-3', and 5'-(6-FAM)-TTCCCAGGACTGAGGAGACGCC-(TAMRA-6-FAM)-3'; murine IL-2, 5'-CCTGAGCAGGATGGAGAAATTACA-3', 5'-TCCAGAACATGCCGACAGAG-3', and 5'-(6-FAM)-CCCAAGCAGGCCACAGAATTGAAAG-(TAMRA-6-FAM)-3'; murine IL-4, 5'-ACAGGAGAAGGGACGCCAT-3', 5'-GAAGCCCTACAGACGAGCTCA-3', 5'-(6-FAM)-TCCTCAGCAACGAAGAACCACCA-(TAMRA-6-FAM)-3'; murine 18 S rRNA, 5'-CCTTTAACGAGGATCCATTGGA-3', 5'-ACGAGCTTTTAACTGCAGCAA-3', and 5'-(6-FAM)-CGCGGTAATTCAGCTCCAATAGCGTATATT-(TAMRA-6-FAM)-3'.

**DNase I Hypersensitivity Assay**—DNase I hypersensitivity assays were performed using isolated nuclei from preactivated T cells as previously described (12). The DNA was digested with NsiI (−6538 and +7125) and analyzed on Southern blots (Nytran Plus, Schleicher & Schuell) using random primer-labeled probes (from +6526 to +7130) and Quickhyb solution (Stratagene).

**Plasmid Constructs**—To generate the wild type (WT) mouse IL-21 promoter luciferase reporter construct, we subcloned the IL-21 gene −1605 to +75 region 5' to the luciferase gene between the BglII and MluI sites in the polylinker of the pGL3-Basic luciferase reporter vector (Promega). Site-directed mutagenesis of the IL-21 NFAT sites in this plasmid was performed using the QuikChange kit (Stratagene). Three mutagenic primers, 5'-CCATGTGTCTCTTTTAAAGGTCTAAGATGCA-GATG-3', 5'-CGTACACCTAGCCAACTTAAAAGAAAAACGAGTTAC-3', and 5'-CACTATACAAAGATTTAAGGGCTGCAATGGGAGGG-3' were used to introduce the indicated underlined 3-bp changes into the NFAT sites at −203, −121, and −76, respectively. Fragments spanning HS2 (+1139 to +1683) were generated by PCR and cloned in both orientations 5' and 3' to the SV40 promoter in pGL3-Promoter.



**A**

Human	GTACCTACTGTTTATTATACATACTTCTTGATTCATGCTGAAAATTGGAATTCACCCAT-	-222
Mouse	ACACACACACACACACACACCCCTTGGTGAATGCTGAAAATTGGAATTCACCCATG	-212
	* * * * *	
	NFAT	
Human	TCTCTCTTTTTCCTGTCAAAGATGCAGATGGGGCACATTTCTGTTGACTCCATCAATCCCT	-162
Mouse	TGTCTCTTTTTCCTGTCTAAGATGCAGATGGGGCACATTTTGTGGACTCTATCCATCCCT	-152
	* * * * *	
	NFAT	
Human	GCCCCACACATTAGCACATGCACACGTATACCTAGCCAGTGGAAAAGAAAAAGAGTTA	-102
Mouse	GCCCCACAC-----GCACACGTACACCTAGCCAATGGAAAAGAAAAACGAGTTA	-102
	* * * * *	
	NFAT	
Human	CTCACATTCATCCATTTTACAAAGATTTCAGGCTGCAATGGGAGGGCTTTACCTCTCCC	-42
Mouse	CTCACACTCATCCACTATACAAAGATTTCAGGCTGCAATGGGAGGGCTTGGCCTCTTCT	-42
	* * * * *	
Human	TGAAGGATGAATAAATAGGTAGCTTAACTGACAACCTGTTCTCAGTCAAGCTGAAGTGAA	+19
Mouse	TGAGGGATGAATAAATAGGTAGCCGTAGCGACAACCTGTGCACAGTCAGGTTGAAGTGAG	+19
	* * * * *	
	NFAT	
Human	AACGAGACCAAGGTCTAGCTCTACTGTTGGTACTTATGAGATCCAGTCCTGGCAACATGG	+79
Mouse	AACCAGACCAAGGCC-----CTGTCATCAGCTCCTG-GAGACTCAGTTCTGGTGGCATGG	+73
	* * * * *	
Human	AGAGGATTGTCATCTGTCTGATGGTCATCTTCTTGGGGACACTGGTCCACAAATCAAGCT	+139
Mouse	AGAGGACCCTTGTCTGTCTGGTAGTCATCTTCTTGGGGACAGTGGCCCATAAATCAAGCC	+133
	* * * * *	

**B**

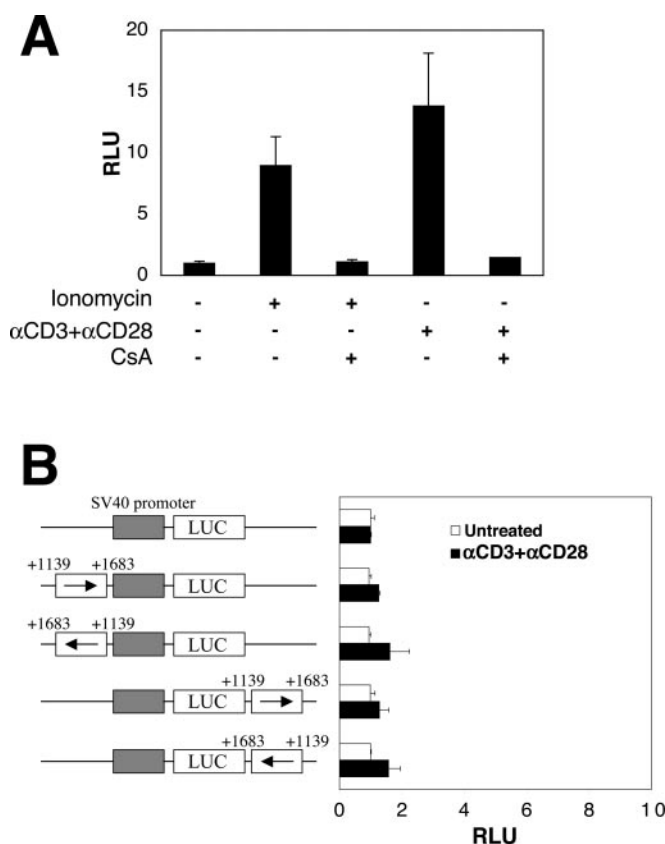
Human	TACCCTGAGAGTATAAATGCAATTGGGGAAATGTTTTCATTGTCACAGACCTTTAATTGC	+1154
Mouse	CTTGGTACCAATGTAAATGAACTGGGGAAATGCTCGCACAGCTA--GGCCTTTAATTGC	+1187
	* * * * *	
Human	ATAAATTAGAATAACTA--AAAGTGCTACCAGTAATTAGGACACTACAAAGGGGGAGACT	+1212
Mouse	ATAAATCGGGAAAACAAGGAGAGTATTGCCAGTAATGAGGGCACCACACAGAG-----	+1240
	* * * * *	
Human	TAAGAAGATACATTGCAAAGAAAACAATTATCGTAAATGACTATGTGTAAAAGTTTAGAA	+1272
Mouse	-----ACACT-CAAAGAAGAGAATTATGGTAAATGAC--AGCGTAAAGTTTAGAA	+1288
	* * * * *	
	AP1	
Human	AGTGAAATGAGTCACAACTATTGCTTTTCTCTAAAATTCAGTCTCTAGCTCTGAACCCAAA	+1332
Mouse	AGTGAGTGAAGTCAAG-CCGCCCTCTGCTCCACAGTTCACTCTCCAGCTCTGCACATGAT	+1347
	* * * * *	
	IRF1	
Human	CACTCTCAT--TTACTAGGTCCTTTTCTAATAAGCAGCATCTAAAATGAAAGTAGCACAT	+1390
Mouse	CATTCTTGTGTTTACTAGGTCCTTT-CAAATGAGTGGCCTCTAAACTGAAAGTAGCTCAT	+1406
	* * * * *	
Human	TCAG-CTTATTGGAAAGACTTTTCCAATAACTGAAAATACTTCTTAATCAATGTGCCTAA	+1449
Mouse	TCAAACCTATTGAAAGGACATTTCGAATAAGTGAGAACATTTTC---TCAGCGTGTCTAA	+1462
	* * * * *	

FIG. 3. **Sequence of human and murine HS1 and HS2.** Sequence comparison of the HS1 (A) and HS2 (B) regions of human and murine IL-21 genes. *Boxed* are potential binding sites for NFAT proteins in the HS1 region, and for AP1 and IRF1 proteins in the HS2 region. In A, the transcription start site of the murine IL-21 gene is *underlined* and indicated by the *arrow*.

**Transient Transfections and Luciferase Assays**—Transient transfections of normal mouse T cells were performed by electroporation, as described previously (13).

**Electrophoretic Mobility Shift Assays (EMSAs)**—CD4<sup>+</sup> splenocytes isolated from C57BL/6 mice were activated with anti-CD3 and anti-

CD28 mAbs for 2 days, and these activated cells were washed and then cultured in the presence of IL-2 for 1 day. Nuclear extracts were prepared as described (14) from these untreated preactivated CD4<sup>+</sup> cells or cells that had been treated with anti-CD3 mAb for 1 h at 37 °C. EMSAs were performed as described previously (13) using 5% polyacrylamide



**FIG. 4. Cyclosporin inhibits IL-21 promoter activity.** A, normal murine T lymphocytes were transfected with a WT HS1 region reporter construct. Transfected cells were incubated with medium, anti-CD3 + anti-CD28 ( $\alpha$ CD3 +  $\alpha$ CD28), CsA, or anti-CD3 + anti-CD28 + CsA. Data are shown as the mean  $\pm$  S.E. B, fragments spanning HS2 (+1139 to +1683) were generated by PCR and cloned in both orientations 5' and 3' to the SV40 promoter in pGL3-promoter. These constructs were transfected into normal murine T lymphocytes and evaluated for their TCR responsiveness.

gels (29:1 acrylamide:bisacrylamide) containing glycerol and 0.5 $\times$  Tris borate-EDTA buffer. For supershifting assays, nuclear extracts were preincubated for 10 min with NFATc1 or NFATc2 mouse mAbs (Affinity Bioreagents, Inc., Golden, CO). Oligonucleotide sequences from the IL-21 promoter and IL-2 distal NFAT binding sites used as probes are shown in Fig. 5A.

**Chromatin Immunoprecipitation**—Chromatin immunoprecipitation assays were performed essentially as described (12, 13). CD4<sup>+</sup> splenocytes isolated from C57BL/6 mice were activated with anti-CD3 and anti-CD28 mAbs for 2 days, and these activated cells were washed and then cultured in the presence of IL-2 for 1 day. These preactivated CD4<sup>+</sup> cells were either left unstimulated or restimulated with 2  $\mu$ g/ml of anti-CD3 mAb for 1 h, followed by cross-linking with formaldehyde. Formaldehyde-treated nuclear lysates were subjected to immunoprecipitation with antibodies specific for NFATc2. After treatment with proteinase K to remove the protein and reversal of cross-links, the amounts of selected DNA sequences were assessed by real time PCR. The sequences of the primers and TaqMan probe for IL-21 promoter were as follows: 5'-GGCACATTTTGTGGACTCTATCC-3', 5'-CCATTG-CAGCCTGGAATCT-3', and 5'-(6-FAM)-AGCCAATGGAAGAAAA-ACGAGTTACTCACACT-(TAMRA-6-FAM)-3'. The sequences of the primers and TaqMan probe for glyceraldehyde-3-phosphate dehydrogenase were as follows: 5'-TTCACCACTGGAGAGAAGGC-3', 5'-GGC-ATGGACTGTGGTCATGA-3', and 5'-(6-FAM)-TGCATCCTGCACCAC-CAACTGCTTAG-(TAMRA-6-FAM)-3'.

## RESULTS

To investigate the regulation of IL-21 expression, we first analyzed IL-21 mRNA levels in CD4<sup>+</sup> T cells. CD4<sup>+</sup> splenocytes isolated from C57BL/6 mice were activated with anti-CD3 and anti-CD28 mAbs for 2 days, washed, and then cultured in the presence of IL-2 for 1 day. These preactivated CD4<sup>+</sup> cells

were then either left unstimulated or restimulated with various stimuli. IL-21 mRNA was increased  $\sim$ 10-fold by anti-CD3 but not by anti-CD28 (Fig. 1A). Co-stimulation with both anti-CD3 and anti-CD28 showed similar effects to those seen with anti-CD3 alone (Fig. 1A). The induction of IL-21 mRNA was primarily at the level of transcription because the transcription inhibitor, actinomycin D, markedly diminished the inducibility (Fig. 1A). This induction did not require *de novo* protein synthesis, because the protein synthesis inhibitor, cycloheximide, did not diminish and in fact increased IL-21 mRNA levels in response to anti-CD3 + anti-CD28 (Fig. 1A). Cycloheximide is a protein synthesis inhibitor that superinduces the expression of many genes by preventing the degradation of otherwise labile mRNAs (15). Interestingly, IL-21 mRNA levels were increased by ionomycin alone, whereas phorbol 12-myristate 13-acetate had little effect by itself and did not augment the effect of ionomycin (Fig. 1A). This is in marked contrast to the regulation of the IL-2 gene in which both phorbol 12-myristate 13-acetate and ionomycin are required for its expression (16)(Fig. 1B). Consistent with the importance of a calcium signal, rapamycin had no effect, but cyclosporin A and FK506 markedly inhibited induction of IL-21 mRNA in response to ionomycin or anti-CD3+anti-CD28 (Fig. 1A); this indicates a role for the calcineurin/NFAT signaling pathway in IL-21 mRNA transcription.

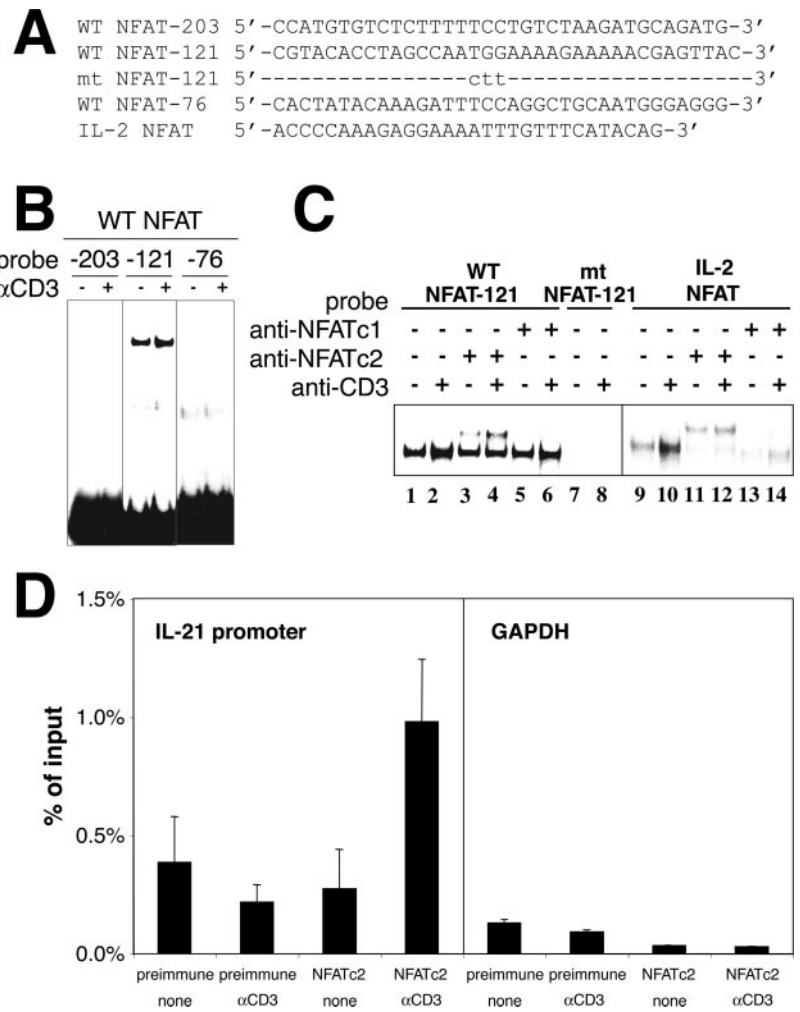
To identify the regions of the IL-21 gene that mediate its induction, we performed DNase I hypersensitivity assays with preactivated CD4<sup>+</sup> T cells that were either unstimulated or restimulated for 1 h with anti-CD3 + anti-CD28. Nuclei were then isolated and subjected to DNase I hypersensitivity assays. DNA was extracted, digested with NsiI, which cleaves the 6.5-kb 5' and 7.1-kb 3' to the transcription start site, and Southern blotted with the indicated probe. Two principal hypersensitive sites, HS1 (upstream of the previously reported 5'-end of the IL-21 cDNA) and HS2 (in intron 2), were identified (Fig. 2). Both HS1 and HS2 were detected in the unstimulated cells, and the intensities of these sites were modestly increased in restimulated cells. The nucleotide sequences of both the HS1 (Fig. 3A) and HS2 (Fig. 3B) regions are well conserved in humans and mice, consistent with the presence of important regulatory regions in these areas.

The transcriptional start site in the IL-21 gene was identified using 5'-RACE and mRNA from activated CD4<sup>+</sup> T cells (data not shown). Using an IL-21-specific primer (see "Materials and Methods"), we identified a single product containing 69 bases of 5'-untranslated mRNA; the 5'-nucleotide was assigned position +1 as indicated in Fig. 3A. The full-length mRNA is thus 16 nucleotides longer than an mRNA previously reported (NCBI accession number NM\_021782). These data confirm that there is not a cryptic upstream exon and that HS1 is located in the proximal 5'-regulatory region.

To test whether HS1 and HS2 correspond to TCR response elements, each HS region was separately subcloned into a luciferase reporter construct. When the HS1 region was used, potent inducibility was observed in response to ionomycin as well as anti-CD3 + anti-CD28 (Fig. 4A), and this inducibility was abrogated when the transfected cells were treated with cyclosporin A 30 min before stimulation (Fig. 4A). In contrast, when the construct containing the HS2 region was cloned in either orientation and either 5' or 3' to the IL-21 or SV40 promoters, only minimal TCR inducibility was found in transfection experiments in normal mouse splenocytes (Fig. 4B). Thus, HS1 appeared to be more important for IL-21 gene regulation, and we have focused on HS1 in this study.

Analysis of HS1 revealed several potential consensus sequences for factor binding, including three consensus NFAT

**FIG. 5. NFAT bind to the IL-21 promoter *in vitro* and *in vivo*.** A, sequence of the -203, -121, -76 (spanning the -216/-182, -136/-101, -89/-55, respectively), and IL-2 NFAT probes. For the -121 probes, both wild type (WT) and mutant (mt) probes are shown. Mutated nucleotides are shown in lowercase, and hyphens indicate identity to the WT sequence. B, EMSA using the -203, -121, and -76 probes in A and nuclear extracts from untreated preactivated mouse T lymphocytes or from cells treated with anti-CD3 ( $\alpha$ CD3) (see "Material and Methods"), as indicated. C, EMSA using the WT and mutant -121 probes and the IL-2 NFAT probe in A and nuclear extracts from untreated mouse T lymphocytes (lanes 1, 3, 5, 7, 9, 11, and 13) or from anti-CD3-treated cells (lanes 2, 4, 6, 8, 10, 12, and 14) in the presence of antibodies to NFATc2 (lanes 3, 4, 11, and 12) or NFATc1 (lanes 5, 6, 13, and 14), as indicated. D, chromatin immunoprecipitation assays reveal inducible NFAT binding to the IL-21 promoter *in vivo*. Murine T lymphocytes were cultured in the absence or presence of anti-CD3 for 1 h, and then chromatin immunoprecipitation assays were performed with preimmune serum or anti-NFATc2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a negative control. Data are shown as the mean  $\pm$  S.E.



binding sites that are conserved in humans and mice (Fig. 3A). We investigated factor binding to  $^{32}$ P-labeled probes spanning these sites using EMSAs and nuclear extracts from unstimulated or anti-CD3-stimulated mouse T cells. When probes spanning the NFAT sites at either -203 or -76 (Fig. 5A) were assayed, only very faint complexes were detected with extracts from either unstimulated or stimulated cells (Fig. 5B). However, the probe spanning the site at -121 (Fig. 5A) generated a complex with extracts from unstimulated cells (Fig. 5, B, lane 3 and C, lane 1), which was modestly increased when cells were stimulated with anti-CD3 (Fig. 5, B, lane 4 and C, lane 2). An antibody to NFATc2 (also known as NFAT1 and NFATp) supershifted the complex to generate a slower mobility complex, with this effect being more evident in activated cells (Fig. 5C, lanes 3 and 4). In contrast, an antibody to NFATc1 (also known as NFAT2 and NFATc) had no effect (lanes 5 and 6). The mutation of the NFAT site eliminated the formation of complexes from resting (lane 7) and stimulated (lane 8) cells.

As a control, we also investigated factor binding to a probe spanning the distal NFAT binding sites in the IL-2 gene. When the IL-2 NFAT probe was used, the formation of complexes was weaker than with the IL-21 probe. Some binding activity was detected in unstimulated cells (lane 9), which increased following stimulation with anti-CD3 (lane 10). The complex formed with the IL-2 NFAT probe was evaluated for the presence of NFATc1 and NFATc2 using antibodies to each protein. An antibody to NFATc2 supershifted the complex to generate a slower mobility complex (lanes 11 and 12), whereas the anti-NFATc1 antibody almost completely blocked the formation of complexes (lanes 13 and 14). Thus, both NFATc2 and NFATc1

proteins can bind to the IL-2 NFAT probe. In contrast, the IL-21 NFAT probe appeared to preferentially bind NFATc2. The fact that much of the complex formed with the IL-21 probe was not supershifted suggested that additional protein(s) contribute to the binding activity seen in unstimulated cells.

We next sought to confirm NFATc2 binding to the IL-21 promoter *in vivo* using chromatin immunoprecipitation assays. Despite *in vitro* binding activity in both resting and stimulated cells, we found a marked increase in NFATc2 binding *in vivo* after cellular stimulation with anti-CD3 (Fig. 5D). No binding of NFATc2 was observed to a control exon region of glyceraldehyde-3-phosphate dehydrogenase gene, indicating the specificity of the binding to IL-21 promoter. Thus, NFATc2 binding *in vivo* corresponded to the induction of *Il21* gene expression.

To determine whether NFATc2 was uniquely essential for IL-21 induction, we next evaluated the ability of anti-CD3 and anti-CD28 to induce IL-21 mRNA expression in NFATc2-deficient mice. Interestingly, IL-21 mRNA induction was not diminished in these mice (Fig. 6). This is analogous to the normal induction of IL-2 mRNA and increased induction of IL-4 mRNA in these animals as well (Fig. 6) (17). The normal induction despite the absence of NFATc2 suggests the compensation by and action of other NFAT family proteins *in vivo*, as has been suggested for the IL-2 and IL-4 genes as well (17).

Despite the normal induction of IL-21 mRNA in NFATc2 knock-out mice, our data overall suggested that the NFAT binding sites were critical. Specifically, the inhibition by cyclosporin A of the induction of IL-21 mRNA (Fig. 1) and promoter activity (Fig. 4) by anti-CD3 and anti-CD28 was consistent with a critical role for the NFAT sites in the HS1 region. To



further define their role, we mutated each NFAT site individually and in combination and assayed the TCR responsiveness of each mutant construct in normal mouse T lymphocytes. Whereas the wild type construct showed ~15-fold TCR inducibility, selective mutation of the NFAT sites at -203 (mutant m1) and -76 (mutant m3) markedly decreased TCR inducibility (Fig. 7), even though these sites individually show little binding by EMSA (Fig. 5B). Selective mutation of the site at -121 most potently bound factors *in vitro* (mutant m2) or double mutation of any two of the sites (mutants m4, m5, and

m6) even more potently decreased TCR inducibility, whereas simultaneous mutation of all three NFAT sites (mutant m7) abrogated TCR inducibility (Fig. 7). These results suggest that all of the NFAT sites are required for maximal TCR inducibility and are consistent with at least some factor binding to all three sites *in vivo*; this binding may be cooperative. By itself, the -121 NFAT site nevertheless appears to be the most important.

DISCUSSION

IL-21 is a type I cytokine that is made by activated T cells and exerts actions on B cells, T cells, and NK cells. Its receptor is most like the IL-2 receptor  $\beta$  chain and the gene encoding IL-21 is adjacent to the *Il2* gene. Similar induction patterns might have suggested similar regulation for these two genes, and our mutagenesis data indicate that the *Il21* gene, analogous to the *Il2* gene, is dependent on NFAT binding sites.

NFAT proteins are expressed in most immune system cells and play a pivotal role in the transcription of cytokine genes and other genes critical for the immune response (18–20). NFAT was first identified in T cells as a rapidly inducible nuclear factor binding to the distal antigen receptor response element of the human IL-2 promoter (21). Subsequently, studies from several laboratories indicated that the promoter/enhancer regions of different activation genes possessed binding sites for NFAT family proteins, including the cytokines IL-2 (22–28), IL-4 (29), IL-5 (30), IL-13 (31), interferon- $\gamma$  (32), tumor necrosis factor- $\alpha$  (33, 34), and granulocyte monocyte colony-stimulating factor (35, 36), as well as the cell surface receptors IL-2 receptor- $\alpha$  chain (13), CD40L (37, 38), and CTLA-4 (39). Based on comparison of these sequences, the NFAT binding site consensus is (A/T)GGAAA(A/N)(A/T/C)N (19).

The activity of NFAT proteins is tightly regulated by the calcium/calmodulin-dependent phosphatase calcineurin, a primary target for inhibition by cyclosporin A and FK506 (19). Calcineurin controls the translocation of NFAT proteins from the cytoplasm to the nucleus of activated cells by interacting with an N-terminal regulatory domain conserved in the NFAT family.

In this study, we have described a TCR response element in the 5'-regulatory region of the *Il21* gene. Several results support the hypothesis that NFAT is required for TCR-mediated transactivation of the *Il21* gene. First, the critical IL-21 promoter region contains three NFAT binding sites, the nucleotide

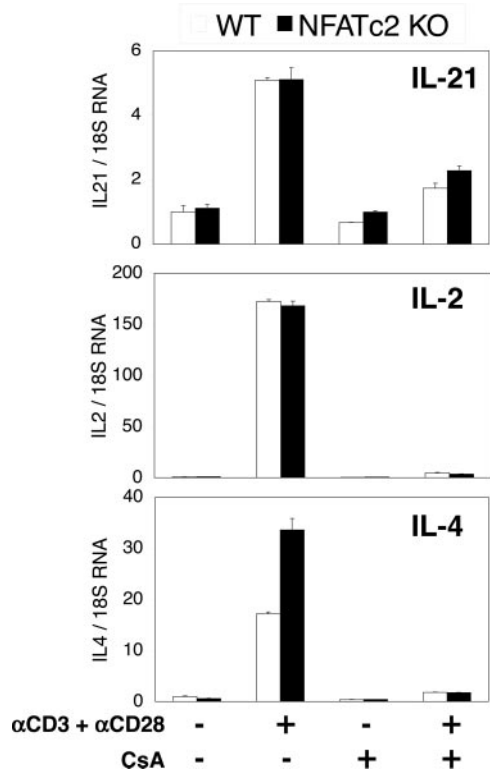


FIG. 6. Normal induction of IL-21 and IL-2 mRNA in NFATc2<sup>-/-</sup> mice. Preactivated murine CD4<sup>+</sup> lymphocytes from WT or NFATc2<sup>-/-</sup> mice were stimulated with immobilized anti-CD3 ( $\alpha$ CD3 +  $\alpha$ CD28) antibody for 4 h, and levels of IL-21, IL-2, and IL-4 mRNA were analyzed by real time PCR. Where indicated, CsA was added 30 min before TCR cross-linking. For each sample, the bar represents the ratio of IL-21, IL-2, or IL-4 mRNA to that of 18 S ribosomal mRNA. Data are shown as the mean  $\pm$  S.E.

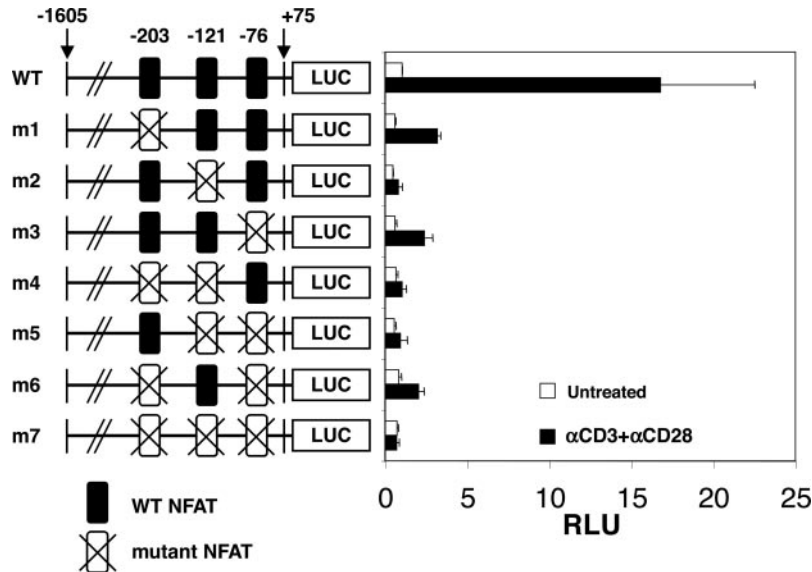


FIG. 7. The NFAT binding sites in the promoter are essential for TCR-induced IL-21 gene expression. Mutations in the NFAT sites were made in the context of the WT -1605/+75 murine IL-21-luciferase reporter construct, as indicated. Constructs were transfected into normal mouse splenic T cells, followed by no stimulation or stimulation with anti-CD3 + anti-CD28 ( $\alpha$ CD3 +  $\alpha$ CD28) for 14 h. Data are shown as the mean  $\pm$  S.E.

sequence of the IL-21 promoter is evolutionarily conserved, and a chromatin immunoprecipitation assay showed the *in vivo* binding activity of NFATc2 to this region. Second, site-specific mutagenesis of these NFAT sites significantly diminished IL-21 promoter activity. Third, CsA, an inhibitor of calcineurin that potently inhibits nuclear translocation of NFAT and NFAT-dependent transcription (19, 40, 41), markedly decreased TCR-mediated transactivation of IL-21 promoter and IL-21 gene expression, implying that calcineurin regulates IL-21 gene transcription. Transient transfection experiments using luciferase reporter constructs showed that CsA could almost completely block anti-CD3 + anti-CD28-mediated IL-21 promoter activity, and this specific role of the NFAT binding sites was confirmed using a construct in which all three NFAT sites were mutated. The fact that NFATc2<sup>-/-</sup> mice had normal IL-21 gene induction is consistent with the potential utilization of more than one NFAT-binding protein *in vivo*.

Recently, Mehta *et al.* (42) reported that NFATc2 and T-bet contribute to Th2-specific regulation of IL-21 expression. They studied IL-21 expression in EL4 and D10 cells, and like us, they found that three NFAT binding sites were important for TCR-stimulated IL-21 promoter activity. We demonstrate in primary T cells the importance of this region and moreover that the chromatin structure in the promoter region has an open conformation. However, our data differ from those of Mehta *et al.* (42) in that we find that IL-21 expression remains strong in NFATc2 knock-out T cells. The basis for this dissimilarity is unclear.

Despite the evidence in support of NFAT recruitment to the *Il2* and *Il4* genes (19, 43) as well as the *Il21* gene, the transcription of the *Il21* gene in preactivated T cells is strikingly different from the genes encoding IL-2 and IL-4 in that it can be mediated by calcium ionophore alone without requiring protein synthesis or protein kinase C activation. Thus, our studies demonstrate that IL-21 induction by antigen in primary T cells is primarily dependent on a calcium signal and contrasts in this regard to these other activation-dependent cytokines that require both calcium and protein kinase C signals (*e.g.* IL-2 and IL-4) or to another activation-dependent protein whose expression only requires a protein kinase C signal (*e.g.* IL-2 receptor  $\alpha$  chain). The lack of a requirement for a protein kinase C signal for IL-21 expression suggests that AP1 family proteins are not important or are much less important for *Il21* gene induction than for induction of the *Il2* gene. It is interesting to speculate that the constitutive DNA binding activity seen even with untreated preactivated cell extracts (Fig. 5B) could contribute to the ability of ionomycin alone to activate the gene. In other words, the constitutive DNA binding activity *in vitro* may correspond to a factor that can cooperate with NFAT proteins *in vivo*, suggesting that the *Il21* gene is poised to receive and respond to a calcium signal as a major component of its regulation. The characterization of such a factor is an interesting area for future investigation. In summary, we have demonstrated properties of regulation of the *Il21* gene and have characterized a critical region containing NFAT binding sites that corresponds to a hypersensitive site, clarifying the molecular basis for the rapid induction of *Il21* gene expression in T cells.

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## REFERENCES

- Leonard, W. J. (2001) *Nat. Rev. Immunol.* **1**, 200–208
- Ozaki, K., Kikly, K., Michalovich, D., Young, P. R., and Leonard, W. J. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 11439–11444
- Parrish-Novak, J., Dillon, S. R., Nelson, A., Hammond, A., Sprecher, C., Gross, J. A., Johnston, J., Madden, K., Xu, W., West, J., Schrader, S., Burkhead, S., Heipel, M., Brandt, C., Kuipper, J. L., Kramer, J., Conklin, D., Presnell, S. R., Berry, J., Shiota, F., Bort, S., Hambly, K., Mudri, S., Clegg, C., Moore, M., Grant, F. J., Lofton-Day, C., Gilbert, T., Rayond, F., Ching, A., Yao, L., Smith, D., Webster, P., Whitmore, T., Maurer, M., Kaushansky, K., Holly, R. D., and Foster, D. (2000) *Nature* **408**, 57–63
- Asao, H., Okuyama, C., Kumaki, S., Ishii, N., Tsuchiya, S., Foster, D., and Sugamura, K. (2001) *J. Immunol.* **167**, 1–5
- Habib, T., Senadheera, S., Weinberg, K., and Kaushansky, K. (2002) *Biochemistry* **41**, 8725–8731
- Kasaian, M. T., Whitters, M. J., Carter, L. L., Lowe, L. D., Jussif, J. M., Deng, B., Johnson, K. A., Witek, J. S., Senices, M., Konz, R. F., Wurster, A. L., Donaldson, D. D., Collins, M., Young, D. A., and Grusby, M. J. (2002) *Immunity* **16**, 559–569
- Mehta, D. S., Wurster, A. L., Whitters, M. J., Young, D. A., Collins, M., and Grusby, M. J. (2003) *J. Immunol.* **170**, 4111–4118
- Ozaki, K., Spolski, R., Ettinger, R., Kim, H. P., Wang, G., Qi, C. F., Hwu, P., Shaffer, D. J., Akilesh, S., Roopenian, D. C., Morse, H. C., III, Lipsky, P. E., and Leonard, W. J. (2004) *J. Immunol.* **173**, 5361–5371
- Ozaki, K., Spolski, R., Feng, C. G., Qi, C. F., Cheng, J., Sher, A., Morse, H. C., III, Liu, C., Schwartzberg, P. L., and Leonard, W. J. (2002) *Science* **298**, 1630–1634
- Suto, A., Nakajima, H., Hirose, K., Suzuki, K., Kagami, S., Seto, Y., Hoshimoto, A., Saito, Y., Foster, D. C., and Iwamoto, I. (2002) *Blood* **100**, 4565–4573
- Zeng, R., Spolski, R., Finkelstein, S. E., Oh, S., Kovani, P. E., Hinrichs, C. S., Pise-Masison, C. A., Radonovich, M. F., Brady, J. N., Restifo, N. P., Berzofsky, J. A., and Leonard, W. J. (2005) *J. Exp. Med.* **201**, 139–148
- Kim, H. P., Kelly, J., and Leonard, W. J. (2001) *Immunity* **15**, 159–172
- Kim, H. P., and Leonard, W. J. (2002) *EMBO J.* **21**, 3051–3059
- Schreiber, E., Matthias, P., Muller, M. M., and Schaffner, W. (1989) *Nucleic Acids Res.* **17**, 6419
- Ragheb, J. A., Deen, M., and Schwartz, R. H. (1999) *J. Immunol.* **163**, 3321–3330
- June, C. H., Ledbetter, J. A., Lindsten, T., and Thompson, C. B. (1989) *J. Immunol.* **143**, 153–161
- Hodge, M. R., Ranger, A. M., Charles de la Brousse, F., Hoey, T., Grusby, M. J., and Glimcher, L. H. (1996) *Immunity* **4**, 397–405
- Crabtree, G. R., and Olson, E. N. (2002) *Cell* **109**, (suppl.) 67–79
- Rao, A., Luo, C., and Hogan, P. G. (1997) *Annu. Rev. Immunol.* **15**, 707–747
- Hogan, P. G., Chen, L., Nardone, J., and Rao, A. (2003) *Genes Dev.* **17**, 2205–2232
- Shaw, J. P., Utz, P. J., Durand, D. B., Toole, J. J., Emmel, E. A., and Crabtree, G. R. (1988) *Science* **241**, 202–205
- Shaw, J., Meerovitch, K., Bleackley, R. C., and Paetkau, V. (1988) *J. Immunol.* **140**, 2243–2248
- Mattila, P. S., Ullman, K. S., Fiering, S., Emmel, E. A., McCutcheon, M., Crabtree, G. R., and Herzenberg, L. A. (1990) *EMBO J.* **9**, 4425–4433
- Randak, C., Brabletz, T., Hergenrother, M., Sobotta, I., and Serfling, E. (1990) *EMBO J.* **9**, 2529–2536
- Brabletz, T., Pietrowski, I., and Serfling, E. (1991) *Nucleic Acids Res.* **19**, 61–67
- Ullman, K. S., Flanagan, W. M., Edwards, C. A., and Crabtree, G. R. (1991) *Science* **254**, 558–562
- Jain, J., Miner, Z., and Rao, A. (1993) *J. Immunol.* **151**, 837–848
- Rooney, J. W., Sun, Y. L., Glimcher, L. H., and Hoey, T. (1995) *Mol. Cell. Biol.* **15**, 6299–6310
- Rooney, J. W., Hoey, T., and Glimcher, L. H. (1995) *Immunity* **2**, 473–483
- Prieschl, E. E., Gouilleux-Gruart, V., Walker, C., Harrer, N. E., and Baumrucker, T. (1995) *J. Immunol.* **154**, 6112–6119
- Dolganov, G., Bort, S., Lovett, M., Burr, J., Schubert, L., Short, D., McGurn, M., Gibson, C., and Lewis, D. B. (1996) *Blood* **87**, 3316–3326
- Campbell, P. M., Pimm, J., Ramassar, V., and Halloran, P. F. (1996) *Transplantation* **61**, 933–939
- McCaffrey, P. G., Goldfeld, A. E., and Rao, A. (1994) *J. Biol. Chem.* **269**, 30445–30450
- Tsai, E. Y., Yie, J., Thanos, D., and Goldfeld, A. E. (1996) *Mol. Cell. Biol.* **16**, 5232–5244
- Cockerill, G. W., Bert, A. G., Ryan, G. R., Gamble, J. R., Vadas, M. A., and Cockerill, P. N. (1995) *Blood* **86**, 2689–2698
- Cockerill, P. N., Bert, A. G., Jenkins, F., Ryan, G. R., Shannon, M. F., and Vadas, M. A. (1995) *Mol. Cell. Biol.* **15**, 2071–2079
- Schubert, L. A., King, G., Cron, R. Q., Lewis, D. B., Aruffo, A., and Hollenbaugh, D. (1995) *J. Biol. Chem.* **270**, 29624–29627
- Tsytysikova, A. V., Tsytikov, E. N., and Geha, R. S. (1996) *J. Biol. Chem.* **271**, 3763–3770
- Perkins, D., Wang, Z., Donovan, C., He, H., Mark, D., Guan, G., Wang, Y., Walunas, T., Bluestone, J., Listman, J., *et al.* (1996) *J. Immunol.* **156**, 4154–4159
- Schreiber, S. L., and Crabtree, G. R. (1992) *Immunol. Today* **13**, 136–142
- Crabtree, G. R. (1999) *Cell* **96**, 611–614
- Mehta, D. S., Wurster, A. L., Weinmann, A. S., and Grusby, M. J. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102**, 2016–2021
- Agarwal, S., Avni, O., and Rao, A. (2000) *Immunity* **12**, 643–652